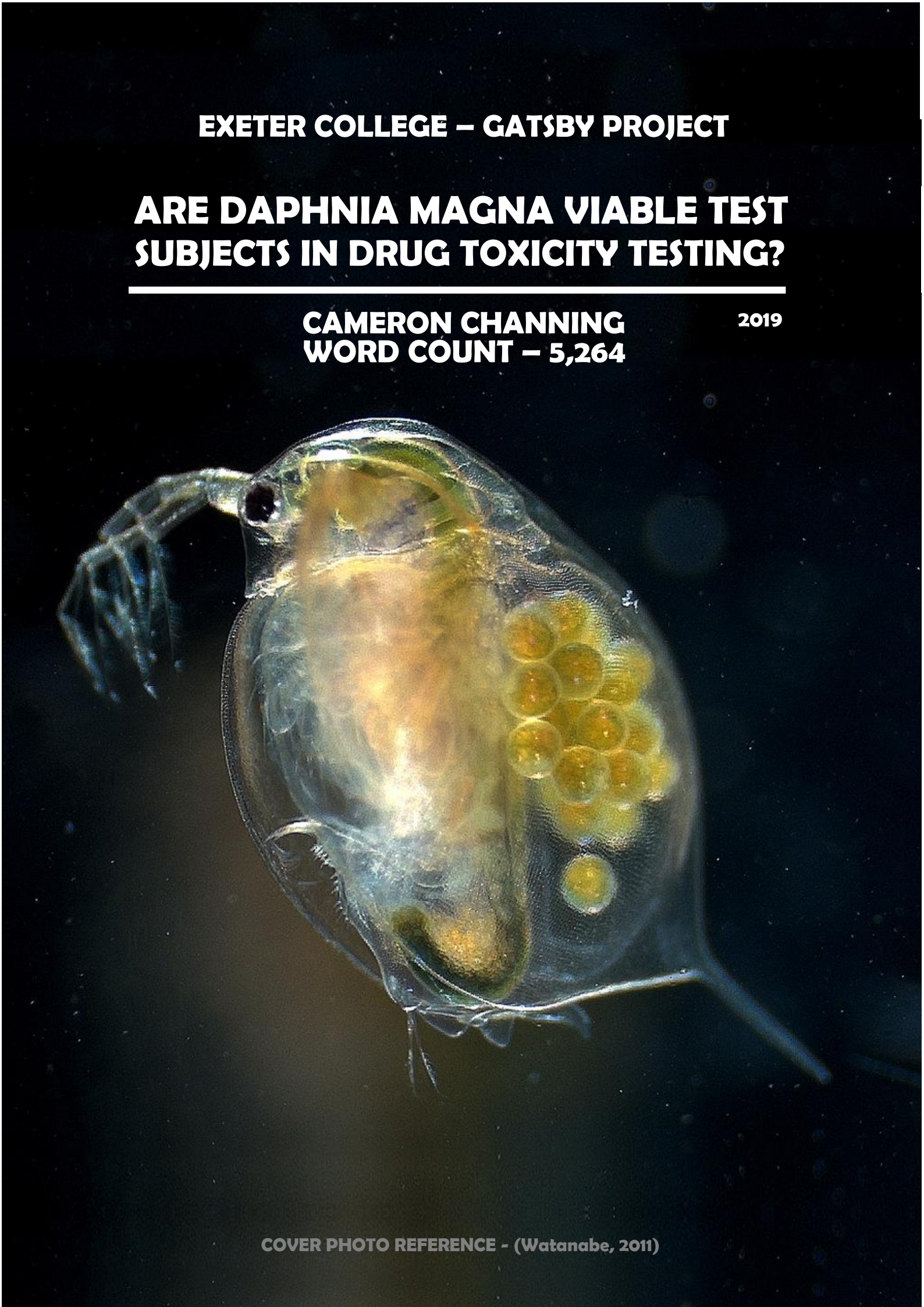
EXETER COLLEGE – GATSBY PROJECT

ARE DAPHNIA MAGNA VIABLE TEST SUBJECTS IN DRUG TOXICITY TESTING?

CAMERON CHANNING
WORD COUNT – 5,264

2019



COVER PHOTO REFERENCE - (Watanabe, 2011)

Contents

Abstract

In this study, Daphnia Magna were tested and observed over a 48-hour period of exposure to a series of paracetamol solutions of varied concentrations to discover if they were viable test subjects when testing a drugs toxicity. The data collected was used to obtain a median lethal dose and to see if they showed a direct correlation between drug concentration and death rate similar to that found in invertebrate toxicity tests. The final estimated median lethal dose was compared against pre-existing data from The European Agency for the Evaluation of Medicinal Products, which showed that the lethal dose of Daphnia was closer to dogs, cats and mice than to that of a human. The data collected showed that there was a direct correlation between the drug concentration and the death rate in the Daphnia, concluding that they are viable test subjects in drug toxicity testing. Although an estimated lethal dose in Daphnia was obtained using the data collected, errors in the testing and anomalies in the data meant that a reliable and exact median lethal dose was inconclusive.

Introduction

Paracetamol, also known as acetaminophen, is one of the most widely used drugs of today (Ther, 2000). Paracetamol is a mild analgesic and antipyretic which is used to help with a plethora of problems in adults and children such as mild to moderate pains, colds, fevers, headaches, migraines and toothaches (NHS, 2016) (DrugFacts, 2017) (BMA, 2018) (Zentiva, 2018). The drug was first used clinically in 1893 by Von Mering but did not appear commercially in the US until 1950 (Ther, 2000) (ACS, 2014) (UKEssays, 2018). Paracetamol was first released onto the United States market under the name Triagesic, a combination of paracetamol, caffeine and aspirin (ACS, 2014) (MigraineMatters, 2016). Triagesic was later removed from the United States market after a report that was released stating that Triagesic was linked to blood diseases, in particular it was said to cause a severe decrease in white blood cell count (MigraineMatters, 2016). Later it was discovered that paracetamol was not the cause of these problems and was released onto the United states market under the brand name “Tylenol”, and was released a year later in 1956 to the United Kingdom in

500mg tablets under the trade name “Panadol” (ACS, 2014) (BMJ, 2015) (MigraineMatters, 2016) (NewWorldEncyclopedia, 2016). Since its initial release it has soared in popularity and is now considered to be a household drug along with aspirin and ibuprofen (NHS, 2017) (FeverMates, 2018).

Paracetamol is usually taken orally and is available to buy over the counter or on prescription in a variety of forms including: tablets, capsules, liquids, suppositories, soluble powders and injection (NHS, 2016) (ADF, 2018). In the United States there are over 600 medicines containing paracetamol making it the most common drug ingredient and widely used drug in the US and the UK (BMJ, 2015) (KnowYourDose, 2017). Paracetamol is most commonly combined with painkillers to increase their effectiveness, it has little known adverse effects and interactions with these drugs making it a safe and inexpensive way to increase their effectiveness (NHS, 2018) (TheRecoveryVillage, 2019). One of the most common paracetamol combinations is co-codamol. Co-codamol tablets contain 500mg of paracetamol and varying doses of codeine, a mild opioid analgesic (BMA, 2018). In the UK 8mg (Codeine) /500mg (Paracetamol) co-codamol tablets are available over the counter (OTC), whilst stronger tablets containing larger doses of codeine are only available on prescription (POM) (Rossiter & Liu, 2017) (NHS, 2019). Other popular combinations include co-dydramol (paracetamol and codeine) and tramacet (paracetamol and tramadol) (NHS, 2018).

Paracetamol remains the leading legal over the counter drug used in suicide attempts and intentional lethal overdoses in the UK (Office for National Statistics, 2017). Drug related suicides are constantly on the rise with a total of 3,774 drug poisoning deaths in the UK in 2016, a 2% increase to the registered drug poisoning deaths in 2015 (Office for National Statistics, 2017). The majority of these deaths are accidental overdoses during the use of recreational drugs such as opiates, amphetamines and cocaine, with intentional overdoses only accounting for about 5%-6% of these overall deaths (Office for National Statistics, 2017). Paracetamol poisoning was been responsible for 1,024 deaths between 2012 to 2016

(6.26% of all drug related deaths) as shown in the table in Appendix A (Office for National Statistics, 2017). The amount of deaths caused by paracetamol led to a reduction in over the counter pack sizes, with a limit of 32 per pack in pharmacies and packs of 16 in non-pharmacy stores (NHS, 2013) (The British Journal of Medicine, 2013). Research taken from recorded statistics of paracetamol deaths and paracetamol-related liver transplants between 1993 and 2009 showed that the new legislation led to a 43% reduction in paracetamol-related deaths and a 61% reduction in people requiring liver transplants due to paracetamol overdose (NHS, 2013) (The British Journal of Medicine, 2013).

The LD_{50} (also known as the median lethal dose) was invented in 1927 by the British pharmacologist John Willian Trevan as a way to estimate the poisoning potency of medicines and drugs (CCOHS, 2018). LD_{50} is an abbreviation for “Lethal Dose 50%” and it involves administering / exposing a population of laboratory animals (common test subjects include mice, rats and guinea pigs) to varying doses of a substance, usually in accordance to their body weight, in almost all LD_{50}’s a pure substance is used instead of mixtures to get more accurate results (CCOHS, 2018). The LD_{50} is the dose in which half of the population exposed to the substance at that dose dies. Drugs can be administered in different forms including feeding the drug by mouth, application of the substance to the skin or injecting it into the veins, body cavity or muscle tissue (Brought to Life, n.d.). The LD_{50} is used as an indicator of a substance’s toxicity, the lower the LD_{50} of a substance the greater its toxicity, the tests in an LD_{50} usually occur for 14 days (Biology Dictionary, 2015) (IAAPEA, 2019).

The tests usually include an average of around 60 subjects of the same species per test, with each test usually undergoing for 14 days (IAAPEA, 2019). Humane killing of the test subjects during the test is avoided as this may interfere with the results (IAAPEA, 2019). Common signs of poisoning experienced by test subjects include tears, diarrhoea, unusual vocalisation, convulsions, discharge and bleeding from the eyes or mouth (IAAPEA, 2019). Pain relief or other drugs are also not given to the subject animals during the tests as this would interfere with the results (IAAPEA, 2019). This method of testing toxicity has been

considered unethical due to allowing the animal to suffer for long periods of times and other reasons mentioned. The LD_{50} is also believed by some to be unreliable as the results in certain animals may not necessarily accurately reflect the lethal dose in humans (Brought to Life, n.d.). The LD_{50} can also be affected by many factors such as animal species, age, sex, diet, food deprivation prior to dosing, season, temperature and experimental procedures (Kuete & Teke, 2014). The variability can be reduced although can never be completely removed / eliminated making the results somewhat less reliable (Kuete & Teke, 2014). Because of the reliability and unethical nature of the LD_{50}, some types of the tests such as oral tests, have been phased out or banned in the UK (Brought to Life, n.d.).

The oral LD_{50} values of paracetamol found in different laboratory animals are shown in the table below (Figure 1).

Animal	Oral LD_{50} value (Mg per kg of body weight)
Rats	2,000
Rabbit	2,000
Guinea Pig	2,000
Dogs	500
Cats	500
Mice	400 – 900

Figure 1 – Oral LD_{50} values of paracetamol in different laboratory animals

Data reference - (The European Agency for the Evaluation of Medicinal Products, 1999)

The worldwide average weight for an adult in 2019 is 136.68lbs (62kg) (Quilty-Harper, et al., 2019) (Roland & Biggers, 2019). By using the oral LD_{50} value of rats from Figure 1 (2,000mg per kg of body weight), the estimated LD_{50} of an average adult is 124,000mg (124 grams), this is the equivalent to 248 paracetamol tablets. Whilst the lethal dose of paracetamol is reported to be very high, the effect on the liver paracetamol causes can be drastic with singular doses as low as 120mg/kg - 7.5 grams (15 tablets) causing hepatotoxicity (liver

damage) in adults (US Pharmacist, 2014) (National Institutes of Health, 2018). Larger doses, or small overdoses causing liver damage left over time can lead to liver failure, which if untreated can cause death. Serious paracetamol overdoses are twice as common in Britain than the rest of Europe and are responsible for 28% of all total liver transplants in the United Kingdom (British Liver Trust, 2017). Although being the most common drug taken in overdoses in the United Kingdom, it is rare for a person to die from paracetamol poisoning if they are admitted to the hospital and given NAC (N-acetylcysteine) within 8 hours of initially ingesting the paracetamol (Bourdeaux & Bewley, 2007). However, a lot of people intentionally do not receive this treatment and even if NAC is administered, liver damage will likely still occur, or the NAC can be unsuccessful in preventing death (Bourdeaux & Bewley, 2007).

Tests in laboratory animals of Aspirin show it to have a 200 mg/kg bw LD_{50} value in rats, although the believed lethal dose is said to be around 500 mg/kg in adult humans (LHS, 2004) (Carter & Nall, 2018). This lethal dose is significantly lower than that of paracetamol, with a lethal dose being 31,000mg (31 grams or ninety-six 325mg tablets) for the average sized person, as opossed to the 124,000mg lethal dose of paracetamol. Although aspirin has a low lethal dose in comparison to paracetamol it is far less likely to be used as a primary drug in suicidal overdoses and is responsible for far less deaths in the UK than paracetamol.

For this experiment Daphnia Magna were used. Daphnia Magna (commonly known as water fleas) are a freshwater zooplankton found in rocky pools and lakes all around the world (Bètadifferentiatie, 2012) (Elenbaas, 2013). Daphnia magna are very commonly used in aquatic toxicity testing due to their extreme sensitivity to changes in their enviroment, they are also easy to culture and due to their clear outer carapace (made mostly of chitin) their internal and external structures (organs) such as their hearts are visible under a microscope, allowing effects to their anatomy be easily monitored (Koivisto, 1994) (Bètadifferentiatie, 2012) (Elenbaas, 2013). Daphnia magna are found in freshwaters and are usually 2mm – 5mm long depending on their age and gender, females are typically larger than the males

with the male daphnia magna typically only growing to 2mm in length (Elenbaas, 2013). Toxicity of a chemical can be monitored by adding it to the Daphnias environment and monitoring changes in heartbeat or by monitoring Daphnia death (Bètadifferentiatie, 2012). When exposed to hypotoxic conditions (low oxygen) they will increase their haemoglobin production, due to their clear outer carapace they will appear more red because of this increase (Bètadifferentiatie, 2012). A labelled diagram of a Daphnia magna and its external and external structures can be seen in Appendix B.

Hypothesis

In pharmocology, a dose response curve is used to show the relationship between the effectiveness of a drug and the amount of drug administered (Study.com, n.d.). Dose response curves are also used to show the effectiveness of a drug against its toxicity (LD_{50} / LC_{50}) to assist in deciding dosage guidelines for the drug. Examples of standard and log-dose response curves can be seen in Appendices C, D, E and F. If values of 100 and 0 are obtainable (0% response and 100% response / 0% mortality and 100% mortality), then a dose response curve can easily be plotted on a graph (GraphPad, 2010).

The hypothesis for this experiment is that the data collected will follow a similar pattern to that of a stanared dose response curve (shown in Appendix C), with the mortality rate per population of Daphnia increasing at a gradual rate as the concentration of the solution is increased. At the lowest concentrations, including the control, no daphnia are expected to die as a result of the paracetamol due to the solutions extremely weak concentration, whereas a 100% mortality rate is expected for the Daphnia exposed to the highest concentrations.

Daphnia are expected to show no or little to no response to the solution within the first data recordings (Immediately after exposure and one hour after exposure), however there should be a visible correlation between solution concentration and mortality rate at the final recording of data (48 hours).

Method

A standard 500ml solution of 0.1 moles per dm^3 (M) was made up boiling 200ml of deionised water at 60°C – 65°C and dissolving the paracetamol powder (The amount added is shown in the calculations below). The paracetamol solution was then filtered whilst still hot to prevent the paracetamol from recrystalising and to filter any additives out. The additives removed during the filtration process included maize starch, croscarmellose sodium and sodium laurisulfate which are listed in the capsules packet leaflet shown in Appendix H.

0.5 litre of 0.1M paracetamol solution $\frac{0.05\ mol\ Paracetamol}{\times 151.165 g/mol\ Paracetamol} / 7.55825 g\ C8H9NO2$

Average capsule contents weight (Calculated using 94 capsules) = 0.515319g

7.55825g (paracetamol required) * 1.030638 (weight of pill to account for additives) = 7.78981966g (7.79g - 2SF) – Weight needed for 0.1M solution

The 200ml solution was placed into a 500ml volumetric flask. The edges of the beaker were washed with deionised water to dissolve any powder / paracetamol collected on the glassware. This was also heated and filtered to remove any additives. This water was then added to the previous 200ml of solution in the volumetric flask. Deionised water was then added to the volumetric flask until the bottom of the meniscus rested on the marked line to ensure an accurate measure of 500ml.

This solution was placed into a burette and mixed with the correct ratio of distilled water, which was also measured in a separate burette, to form different concentrated solutions of 50mls for testing including 0.05 M, 0.025 M and 0.0125 M.

50ml from the original 0.1 moles per dm^3 (M) was placed into a separate 500ml volumetric flask, deionised water was added to the flask until the bottom of the meniscus was level with the 500ml line to produce a 0.01 M. This new solution was also placed into a burette and mixed with deionised water in different ratios to produce more 50ml samples for testing including concentrations of 0.005 M and 0.0025 M.

The same method shown above was used to produce a 0.001 M solution from the 0.01 M solution. This solution was again measured using a burette and mixed with different ratios of deionised water to produce 50ml samples of different concentrations to include 0.0005 M, 0.00025 M, 0.000125 M and 0.0001 M.

Approximately 20-30 Daphnia were collected at a time using a pipette and placed in a separate beaker of deionised water. Once the required amount of Daphnia was collected in the water, they were collected and removed using a strainer. The strainer was turned over and a solution was poured over the strainer, removing the Daphnia from the strainer and moving them into a new beaker containing 50ml of the concentrated solution poured over them. This method was used and repeated for each different concentration tested.

The daphnia were handled with caution and care to avoid any unnecessary stress. They were only exposed to the solutions for the minimum necessary time required to show results / change and collect the data. Over exposure of the daphnia was avoided to reduce the stress caused to the daphnia and they were stored in an area away from harsh sunlight at room temperature to avoid and extra unneeded stress when they were not being monitored. Sudden movements and over interference to their environment was also avoided whilst they were being monitored and data was being collected.

The independent variable in the experiment was the solution concentration, with the dependent variable being the health and fatality rate of the Daphnia Magna. Controlled variables included the volume of solution to which each group of Daphnia was exposed to and the environment of the Daphnia before and during experimentation.

Risk Assessment

Hazard	Risk	Control	P	S	Risk Level	Further Action
Broken glass	Cuts, scrapes and puncture wounds from sharp edges or glass fragments from beakers and glass equipment.	Ensure equipment is in a safe a suitable set up and avoid harsh handling of glass.	1	2	L	Inform the instructor leading the experiment if glass is broken.
Slippery surfaces	Slips and trips from spilled water or solutions from taps or beakers.	Ensure that all equipment is arranged in a safe setup to avoid beakers falling or spilling.	3	2	L	Clean up any spilled water from all surfaces as soon as it is spilled.
Burns from Bunsen burner	Direct contact with the flame of the Bunsen burner or hot metal heated by the Bunsen burner such as a tripod or gauze can cause burning of the skin.	Avoid directly touching the flame and handle the Bunsen burner using its base. Allow metals to cool after being close to / heated by the Bunsen burner. Take extra precaution when using the blue flame.	2	2	L	Treat any burns with cold water and inform the lecturer leading the experiment.
Heated water	Burns or scolding of the skin from boiled water or contact with the heated beaker.	Allow the boiled water and heated beakers to cool before directly touching either of them. If hot water needs to be transferred, then use tongs or other safety equipment.	1	2	L	Treat any burns with cold water and inform the lecturer leading the experiment.
Allergic reactions / Skin irritation from paracetamol capsule contents	Allergic reactions or skin irritation from contact with the paracetamol capsules contents or Daphnia Magna.	Avoid direct contact, especially contact to the eyes and mouth with any potentially allergic substances such as paracetamol and Daphnia. Wear safety equipment to minimise contact and inform the instructor leading the experiment of any allergies prior to the experiment.	1	1	L	If irritation of the skin becomes apparent, then wash / rinse area with water.
Tripping hazard from chairs and equipment	Slips, trips and falls from equipment or chairs left out.	Ensure all chairs are tucked in and any other equipment is not left out on the floor.	3	2	L	
Irritation of the eyes from capsule powder	Irritation of the eyes from paracetamol powder capsule contents	Wear safety glasses when opening, weighing and handling capsules and the powdered contents. Wash hands after handling of paracetamol and avoid touching face / eyes after handling the powdered mixture.	1	1	L	
Bacteria transfer from Daphnia Magna	Possible bacteria can be transferred or picked up from Daphnia.	Avoid contacting eyes and mouth when handling daphnia. Wear safety equipment and use equipment when transferring daphnia to minimise contact and transfer of bacteria.	1	1	L	

Hazard severity (S)	Probability of occurrence (P)	Risk Level (PL)
1 – None 2 – Minor Injury 3 – Major Injury 4 – Major Injury / Death	1 – Very Unlikely 2 – Unlikely 3 – Likely 4 – Very Likely	Low (L). 1-6 Medium (M). 7-10 High (H). 11-16

Results

The raw data collected from the experiment is shown in Appendices I through to L. The processed data, showing all collected observations and mortality rates of the Daphnia is shown in appendix M. The individual tables showing the fatality percentage of each Daphnia population per solution at different time intervals is shown in Appendix N, with the solution concentrations converted to milligrams per 50ml. Outliers and inconsistent data results are highlighted in red in Appendix O. All other results and processed data is shown and labelled below in Figures 2-15.

1 Hour After Exposure	
Dosage (Mg per 50ml)	***Population Deceased (%)***
0.0	0
755.8	83
377.9	56
189.0	32
94.5	0
75.6	0
37.8	0
18.9	0
7.6	0
3.8	0
1.9	0
0.9	0
0.8	0

Figure 2 – Table showing the percentage of Daphnia population deceased from each solution after 1 hour. Solution concentration converted to milligrams per 50ml.

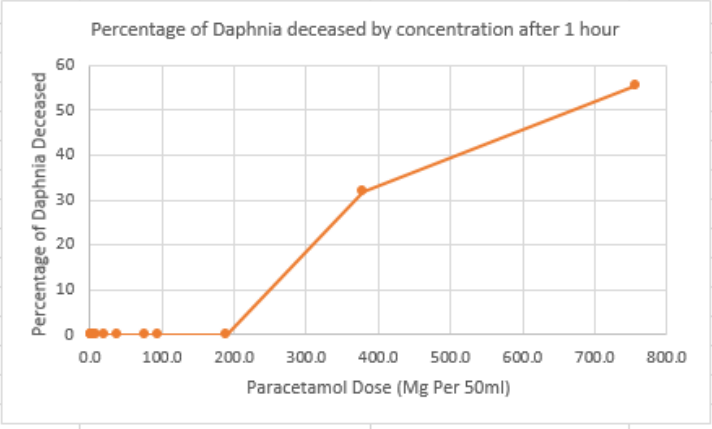


Figure 3 – Line graph plotted from the data shown in figure 2.

3 Hours After Exposure	
Dosage (Mg per 50ml)	***Population Deceased (%)***
0.0	5
755.8	100
377.9	100
189.0	84
94.5	35
75.6	17
37.8	7
18.9	0
7.6	0
3.8	0
1.9	0
0.9	0
0.8	4

Figure 4 – Table showing the percentage of Daphnia population deceased from each solution after 3 hours. Solution concentration converted to milligrams per 50ml.

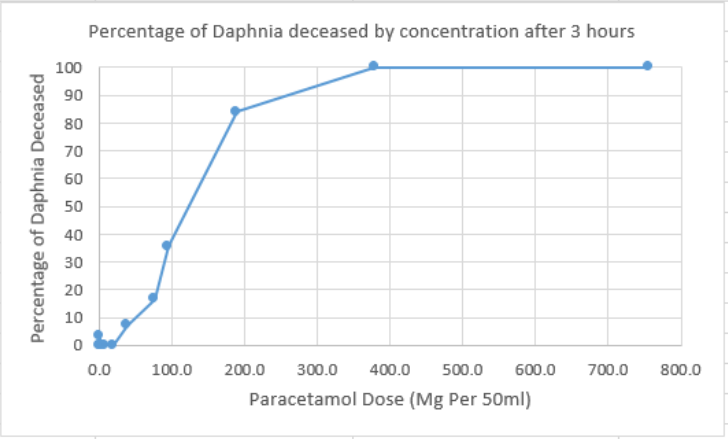


Figure 5 – Line graph plotted from the data shown in figure 4

48 Hours After Exposure	
Dosage (Mg per 50ml)	Population Deceased (%)
0.0	10
755.8	100
377.9	100
189.0	100
94.5	100
75.6	100
37.8	71
18.9	66
7.6	68
3.8	49
1.9	35
0.9	53
0.8	11

Figure 6 – Table showing the percentage of Daphnia population deceased from each solution after 48 hours. Solution concentration converted to milligrams per 50ml.

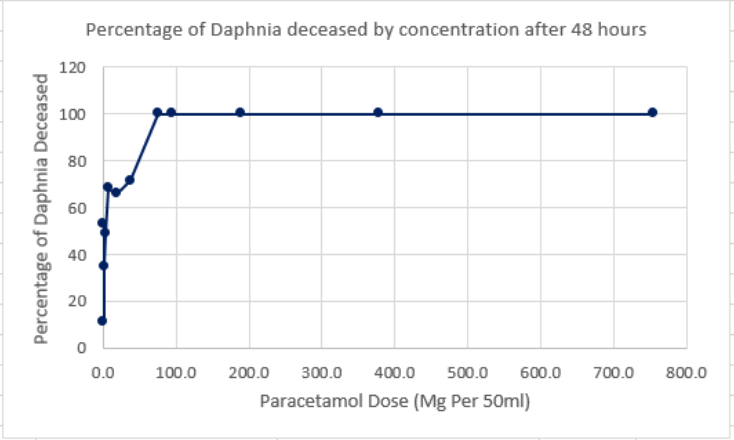


Figure 7 – Line graph plotted from the data shown in figure 6

48 Hours After Exposure	
Dosage (Mg per 50ml)	Population Deceased (%)
0.0	10
755.8	100
377.9	100
189.0	100
94.5	100
75.6	100
37.8	71
18.9	66
7.6	68
3.8	49
1.9	35
0.9	53
0.8	11

Figure 8 – Outliers highlighted in red of data shown in figure 6.

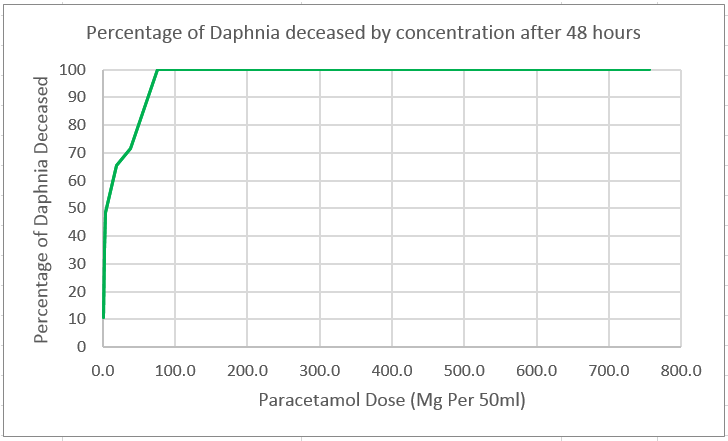


Figure 9 – Line graph plotted from the data shown in figure 6 with the highlighted outliers removed.

48 Hours After Exposure	
Dosage (Mg per 50ml)	Population Deceased (%)
37.8	71
18.9	66
3.8	49
1.9	35

Figure 10 – Table showing the four solutions closest to a 50% fatality rate of the Daphnia after a 48-hour exposure, excluding the outliers shown in figure 8.

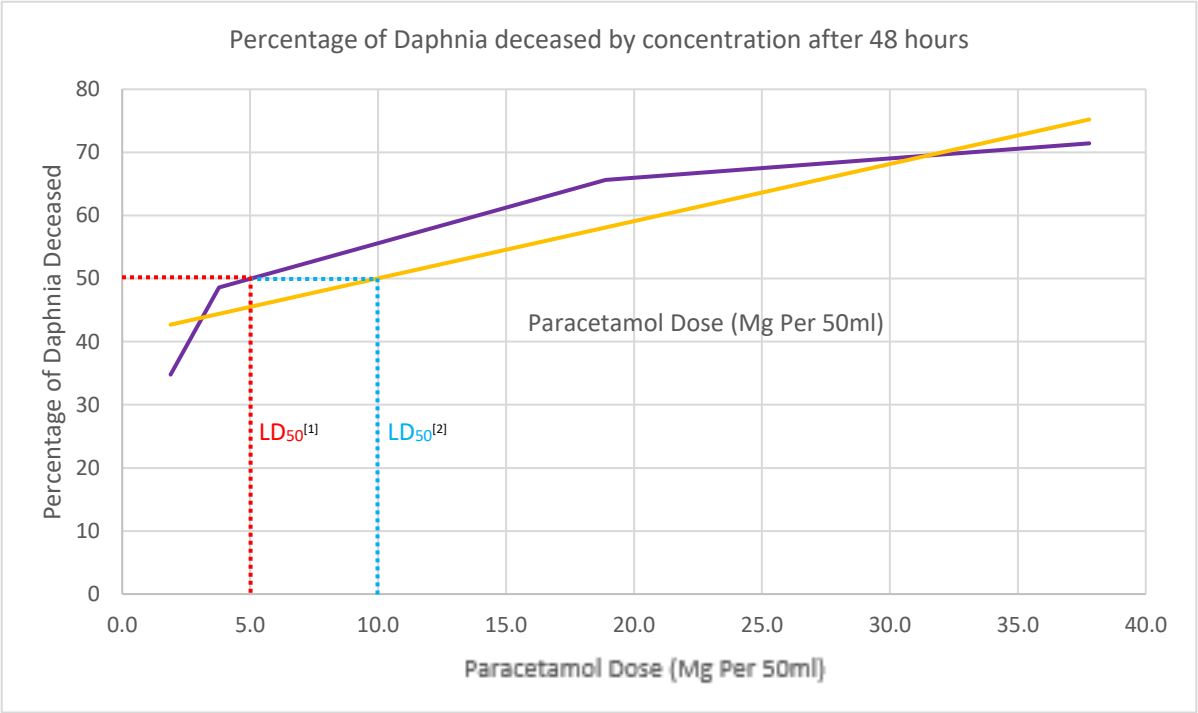


Figure 11 – Line graph plotted from the data shown in figure 10 with a line of best fit shown in orange with two possible LD_{50} values labelled.

48 Hours After Exposure	
Dosage (Mg per 50ml)	***Population Deceased (%)***
37.8	61
18.9	56
3.8	39
1.9	25

Figure 12 – Table with the data from figure 10, with the percentage of deceased daphnia reduced by 10% per test to accommodate for the natural mortality rate over time shown in the control.

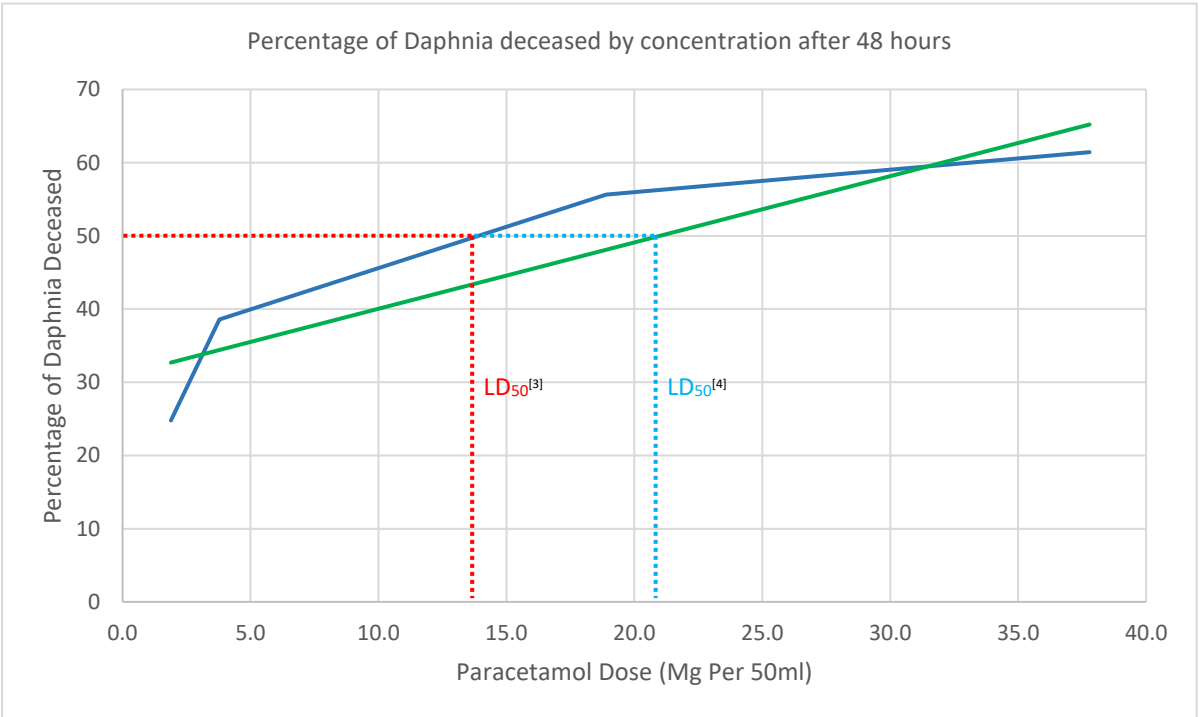


Figure 13 – Line graph plotted from the data shown in figure 12 with a line of best fit shown in green, with two possible LD_{50} values labelled.

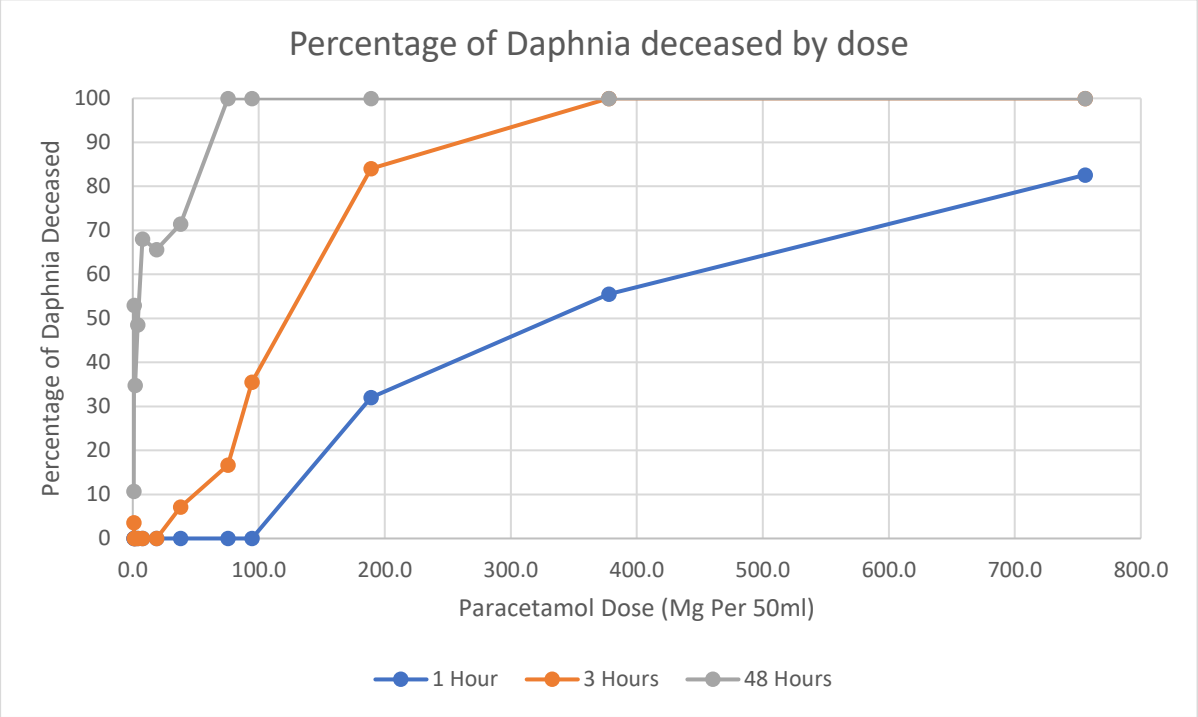


Figure 14 – Line graph showing original total percentages of Daphnia deceased by dosage after 1 hour, 3 hours and 48 hours of exposure.

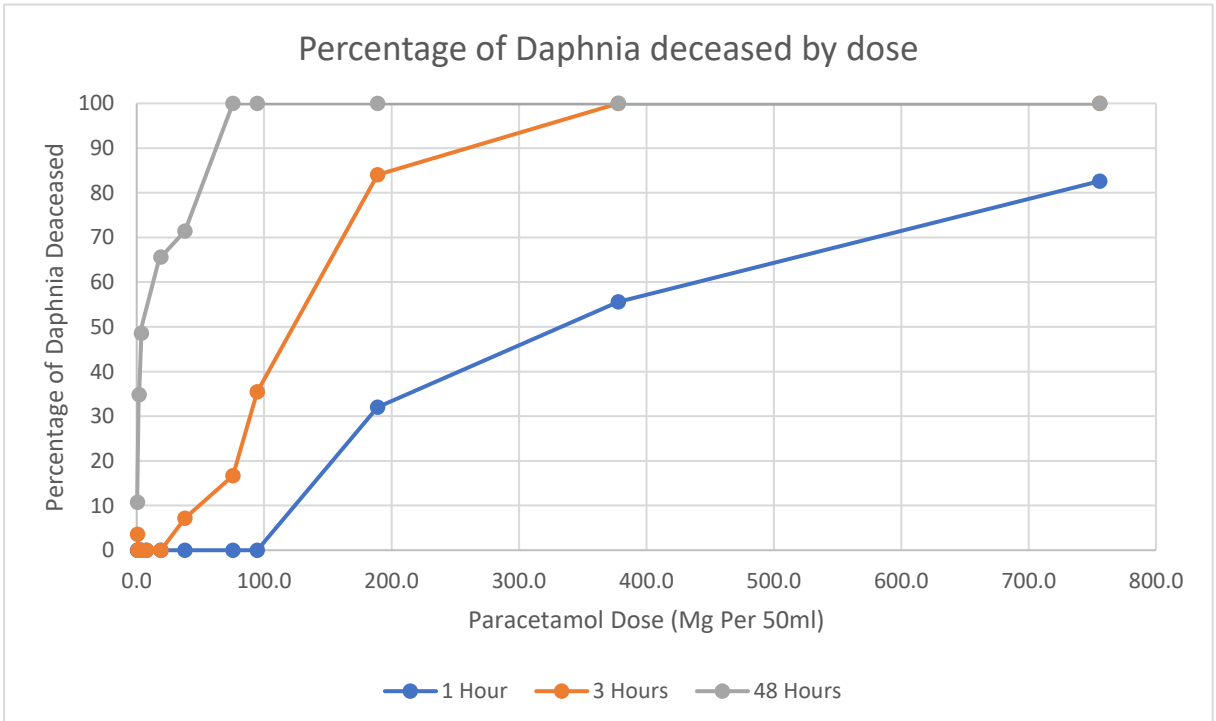


Figure 15 – Line graph showing total percentages of Daphnia deceased by dosage after 1 hour, 3 hours and 48 hours of exposure (Outliers highlighted in figure 8 removed).

Analysis

The results taken from the experiment showed that the Daphnia Magna quickly responded to high doses of paracetamol. With the data collected and displayed in Appendices J and M showing that the Daphnia which were exposed to concentrations equal to and higher than 0.025 Moles per dm^3 (94.5 mg per 50ml) began to succumb to paracetamol toxicity within the first hour of exposure. The daphnia who were exposed to the most concentrated solution (0.1 Mole per dm^3 / 755.8 mg per 50ml) showed the highest mortality increase of all collected data within the 48 hours, with 83% of Daphnia becoming deceased within the first hour of exposure. Observations of the Daphnia showed that Daphnia who were exposed to solutions of 0.01 Moles per dm^3 (M) and higher began to display adverse effects such as delayed, slow and inconsistent movements as a result of the paracetamol within the first hour of exposure. Daphnia that were exposed to solutions less than 0.01 M displayed no observable adverse effects within the first hour of exposure. In total, 56% of all Daphnia exposed to solutions equal to or higher than 0.025 M died within the first hour. This data shows that Daphnia may respond and be adversely affected by paracetamol faster than humans. Paracetamol overdose patients are commonly asymptomatic within the first 24 hours of an overdose, whilst the data collected shows that the effects of paracetamol became deadly within the first hour to a significant percentage of Daphnia (Knott, 2019) (Doctors.net, n.d.). Mortality percentages of the Daphnia after the first hour of exposure were plotted and shown on the graph in Figure 3, the graph shows that the mortality rate of Daphnia increased at a relatively gradual rate as the dose was increased, this relationship between dose and mortality reflects that of which was hypothesised.

After the second set of data was collected (1 hour after exposure), the Daphnia were placed back into their original environment away from harsh sunlight. After two more hours the Daphnia were observed with a magnifying glass and observations of their movements and additional mortalities were taken, the data collected is shown in Appendix K, M and Figure 4. The data collected from the third observation showed that Daphnia exposed to the most

concentrated solutions were significantly affected by the paracetamol, with fatality rates of Daphnia exposed to solutions equal to or higher than 0.025 M rising from 56% after one hour to 95% after 3 hours. Daphnia exposed to the highest concentrations (0.1 M and 0.05 M) showed a 100% mortality rate as a result of the paracetamol solution, further supporting that Daphnia are adversely affected by paracetamol faster than humans.

After three hours, the control group and the test group exposed to the weakest solution (0.0001 M) both showed a singular fatality, with the rest of the group showing no visible changes in behaviour or movement. This may suggest that some of the Daphnia tested were not of great health.

Daphnia exposed to solutions as weak as 0.001 M showed visible changes in their behaviour and movement as a result of the paracetamol, whereas all Daphnia exposed to solutions equal to or less than 0.0005 M showed no response to the paracetamol after 3 hours of exposure. The percentage of mortalities per group were calculated and presented in a table against the dosage they were exposed to, this table can be seen in Figure 4. This data was then plotted and shown as a graph in Figure 5. The graph shows that there is a noticeable relationship between the mortality rate of Daphnia and the dose of paracetamol. The graph follows a similar relatively gradual relationship to that discussed previously in Figure 3 however, Figure 5 shows a steeper incline, suggesting that the Daphnia has a far greater response to the paracetamol after 3 hours than after the initial 1 hour. Figure 5 shows a similar trend to that which was previously hypothesised, with a relatively gradual increase in deaths per population with increasing dosage, similar to that of the standard dose response curve shown in Appendix C. Using the line graph in Figure 5, it can be calculated that the median lethal dose (LD_{50}) in Daphnia from paracetamol exposure after three hours is around 120mg-125mg per 50ml of solution (2,400mg/L – 2,500mg/L).

Once the Daphnia had been observed and the data had been recorded, the Daphnia were again placed into their original environment away from harsh sunlight. Due to time complications, the Daphnia were unable to be observed after 24 hours and were instead

observed after 48 hours of exposure. After 48 hours from their initial exposure, the Daphnia were collected and observed again using a magnifying glass, observations and fatalities of the Daphnia were recorded for the final time before the Daphnia were safely disposed of. The raw collected data after 48 hours is shown in Appendix L and can be seen in the completed observation table in Appendix M.

The observations recorded and shown in both Appendices L and M shows that the Daphnia became extremely responsive to paracetamol after 48 hours of exposure, with every group of Daphnia showing fatalities. In total, 70% of all Daphnia who were exposed to paracetamol had died by the time of final recording, with 43% of all Daphnia dying between 3 hours and 48 hours of exposure.

The data collected after 48 hours of paracetamol exposure showed that paracetamol was lethal to all Daphnia in concentrations of 0.01 M or higher. Daphnia exposed to concentrations of paracetamol that were 0.00025 M or higher displayed adverse behaviour as a result of the paracetamol such as slow movements and long, moderate or short periods of no movement. At least every test group of Daphnia, including the control, suffered fatalities by the end of the 48-hour test period.

The percentage of overall fatalities per group was added to a table against the dosage they were exposed to, this table is shown in Figure 6. This data was plotted on a graph which can be seen in Figure 7.

The data collected at the 48-hour mark showed some clear discrepancies and inconsistencies that were not present in previous data recordings, these inconsistencies are highlighted in red in both Appendix O and Figure 8.

The groups of Daphnia exposed to 0.001 M (7.6 mg per 50ml) and 0.000125 M (0.9 mg per 50ml) showed clear anomalies that was not consistent with the rest of the data collected. The daphnia exposed to the solution containing a total of 7.6 milligrams had a fatality rate of 68% after 48 hours of exposure, this percentage was higher than that of the group exposed

to the more concentrated solution (18.9 mg per 50ml / 0.0025 M). Despite being over 2.5 times greater in concentration, the 18.9 milligram solution had a 2% lower fatality rate. The daphnia exposed to the solution containing 0.9 mg of paracetamol showed a similar inconsistency, with the fatality rate of Daphnia being 18% greater than those Daphnia exposed to the more concentrated solution (1.9 mg per 50ml / 0.00025 M).

There are many possible theories to what caused these irregularities. The most probable cause being human error. When looking at the data shown in Appendix O, it is noticeable that the fatality rates for each group of Daphnia descend at a relatively gradual rate in accordance to the dosage they were exposed to, this may suggest that the outliers had been exposed to solutions that were more concentrated than intended. It is possible that these solutions were made incorrectly by using imprecise or incorrect ratios of distilled water and concentrated paracetamol solution. Due to the precise ratios required, any small errors in making the solutions could have led to a much stronger or weaker solution than intended.

Another plausible cause of these outliers may have been due to crystallised paracetamol sinking to the bottom of the burette when preparing the solutions. When making the solutions there was occasional breaks to refill burettes and calculate the correct ratios required for solutions. Long breaks may have caused some of the paracetamol in the solution to crystallise and fall to the bottom of the burette it was in, causing the final solution to be more concentrated than anticipated.

The last possible cause of these outliers is the Daphnia used. Some groups of Daphnia were selected from different batches due to the limited number of healthy Daphnia in the batch used for most other solutions. The Daphnia used for testing in these two concentrations may have been from a batch containing Daphnia who were less healthy due to complications in their environment or due to how they were stored and handled.

To achieve a higher level of accuracy, these groups were removed from conclusive data. Figure 9 shows the percentage of overall fatalities per group against their exposed dosages

with these outliers removed. Figure 9 shows that there is an obvious relationship between fatality rates of the Daphnia and the dosage they were exposed to, with the graph showing that the death rate per group of Daphnia increased at a relatively gradual rate as the dosage was increased.

To obtain a more accurate LD_{50}, the four solutions which were the closest to a 50% death rate after the 48 hours were selected (shown in Figure 10) and plotted as a separate graph which can be seen in Figure 11. A line of best fit was added to show the general trend, two LD_{50}'s are shown on this graph (labelled $LD_{50}^{[1]}$ and $LD_{50}^{[2]}$). The LD_{50}'s were taken from the points at which the trend line and the original line intersected with the 50% mark. The original line intersects the 50% mark at 5mg ($LD_{50}^{[1]}$) and the trend line intersects at 10mg ($LD_{50}^{[2]}$). It is estimated that the LD_{50} sits closer to 10mg than 5mg as $LD_{50}^{[1]}$ only shows the LD_{50} based on the two sets of data closest to achieving a 50% death rate (18.9 mg per 50ml and 3.8 mg per 50ml), whereas $LD_{50}^{[2]}$ shows the median lethal dose based on the general trend between the mortality percentage and the dose.

The Daphnia in the control group suggested that some of the Daphnia may have died over the 48-hour testing period due to natural causes. In total, 10% of the Daphnia in the control group had died at the time of final recording. The Daphnia exposed to the weakest solution (0.0001 M) did not appear to be affected by the paracetamol solution but showed similar results, with 11% of Daphnia being dead at the time of final recording, further supporting the idea that some of the test subjects had died as a result of natural causes and not from the paracetamol. To accommodate for this, the percentages of deceased Daphnia were reduced by 10% (as shown in Figure 12). The graph in Figure 13 shows the two LD_{50} values after accommodating for this natural death rate. The original line now intersects the 50% mark at 13.5mg ($LD_{50}^{[3]}$) and the trend line intersects at 21.5mg ($LD_{50}^{[4]}$). The LD_{50} values taken from the trend lines in both Figures 11 and 13 ($LD_{50}^{[2]}$ and $LD_{50}^{[4]}$) are more reliable due to following the trend / relationship between paracetamol dose and fatality percentage of Daphnia. From this data it is estimated that the median lethal dose of Daphnia for

paracetamol is between 10mg and 21.5mg per 50ml solution, however due to discrepancies in the data and errors during testing an exact value is inconclusive.

Evaluation

Overall, the test provided some usable data. Although the data showed some irregularities, once removed the data helped in showing that there is a clear relationship between the death rate of Daphnia and the dosage to which they were exposed to. However, there were obvious limitations when designing the experiment such as equipment, time and additional resources which may have had an impact on the overall results, including the explained inconsistencies in some of the data.

More accurate ways of measuring the 50ml solutions such as volumetric flasks could have been used, this may have reduced the chances of having inconsistencies in the data, however due to limited resources and time this was not feasible during this experiment. Due to time restraints and the time required to make the solutions, a large percentage of the Daphnia ordered for testing had deteriorated in health, with many of the Daphnia dying before they were added to the solutions. Healthier Daphnia may have reduced the likelihood of some irregularities presented in the collected data and may have helped achieve a more accurate result. Time complications also led to the original method being changed, with the final observations of the Daphnia being taken after 48 hours as opposed to the originally planned 24 hours. Due to limited time and resources, only a singular trial was done. Multiple trials would have helped in achieving a more accurate and conclusive result as well as in identifying errors during testing. More precise methods and equipment used for counting and observing the Daphnia could have also been used to reduce human error when counting fatalities.

Discussion

Overall, the experiment did not go as was originally planned. Changes were made to accommodate for time restraints and the limited equipment available. Due to the experiment

only consisting of a singular trial and anomalies in data, the results are not viable as reliable scientific data and therefore the LD_{50} of paracetamol in Daphnia was inconclusive. Anomalies in the data deemed only a portion of the originally planned tests were usable for data, these anomalies were believed to be caused by human error or by fault of the equipment or test subjects used, however due to the limited number of trials the direct cause of these inaccuracies was not determined. Since the dosage was measured in milligrams per 50ml of solution, an exact conversion of milligrams per body weight of the Daphnia was not achieved, the closest determined LD_{50} was believed to be between 200mcg/ml – 430mcg/ml (200mg/L – 430mg/L).

Conclusion

In conclusion, whilst the data obtained was deemed unreliable due to errors in testing and limited trials, it did show that there was a clear relationship between the number of fatalities and the dose of paracetamol to which the Daphnia were exposed to, suggesting that Daphnia may be viable test subjects when testing drug toxicity. The usable data did also in ways represent the pattern seen in a standard dose response curve as was hypothesised.

The data also showed that Daphnia may respond to paracetamol quicker than humans, with a large percentage of Daphnia becoming deceased within the first three hours of exposure, whereas humans are usually asymptomatic during the first 24 hours of an overdose (Knott, 2019) (Doctors.net, n.d.).

Since the dosage was measured in milligrams per 50ml of solution, an exact conversion of milligrams per body weight of the Daphnia was not achieved. The closest determined LD_{50} was said to be between 200mg/L and 430mg/L (200mcg/ml and 430mcg/ml) which is similar to the LD_{50} values of mice, dogs and cats collected from The European Agency for the Evaluation of Medicinal Products (shown in Figure 1), however as previously discussed the anomalies in the data, lack of multiple trials errors during testing caused this data to be deemed unreliable and therefore a conclusive value was not achieved.

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APPENDIX A

Data collected showing the amount of deaths caused by commonly overdosed drugs in the UK in between 2012 and 2016 (Office for National Statistics, 2017).

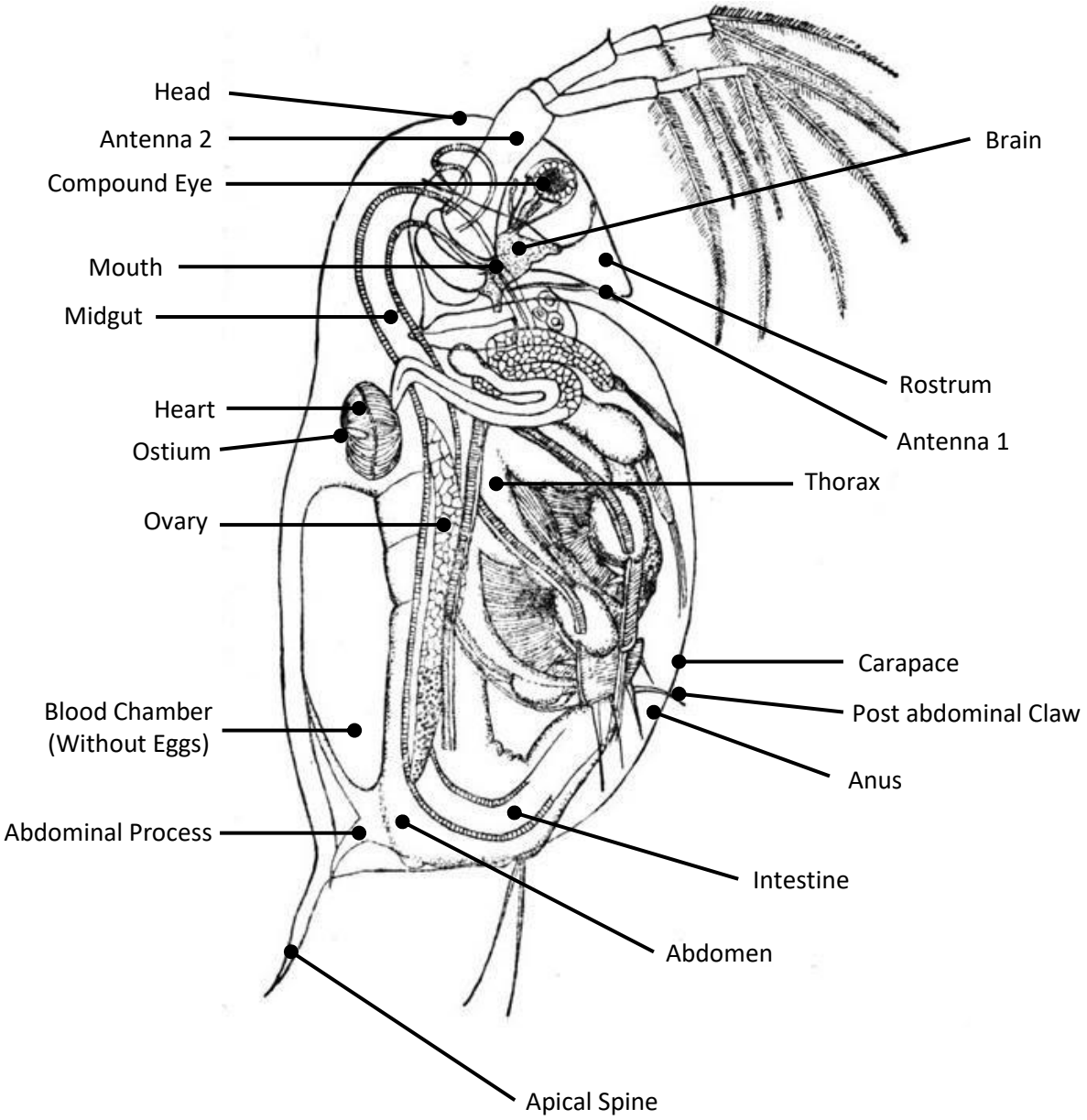
Number of deaths

	2012	2013	2014	2015	2016
All drug poisoning deaths	2,597	2,955	3,346	3,674	3,744
Any opiate[4]	1,290	1,592	1,786	1,989	2,038
- Heroin and/or morphine	579	765	952	1,201	1,209
- Methadone	414	429	394	434	413
- Tramadol	175	220	240	208	184
- Oxycodone	37	51	51	51	75
- Fentanyl	22	22	40	34	58
Cocaine	139	169	247	320	371
Any amphetamine	97	120	151	157	160
Any new psychoactive substance	55	63	82	114	123
Any benzodiazepine	284	342	372	366	406
Pregabalin	4	33	38	90	111
Gabapentin	8	9	26	49	59
All antidepressants	468	466	517	447	460
Paracetamol[5]	182	226	200	197	219
Propranolol	39	46	54	55	45

The above table shows deaths caused by both intentional and unintentional overdoses of illegal and legal substances in the UK between 2012 and 2016. Overall 16,356 overdoses were recorded within these four years with opiate drugs such as heroine being the highest and most lethal. Within these four years a total of 1,024 paracetamol overdose deaths were recorded equating for 6.26% of all overdose related deaths. This makes paracetamol the most common over the counter medication used in deadly overdoses.

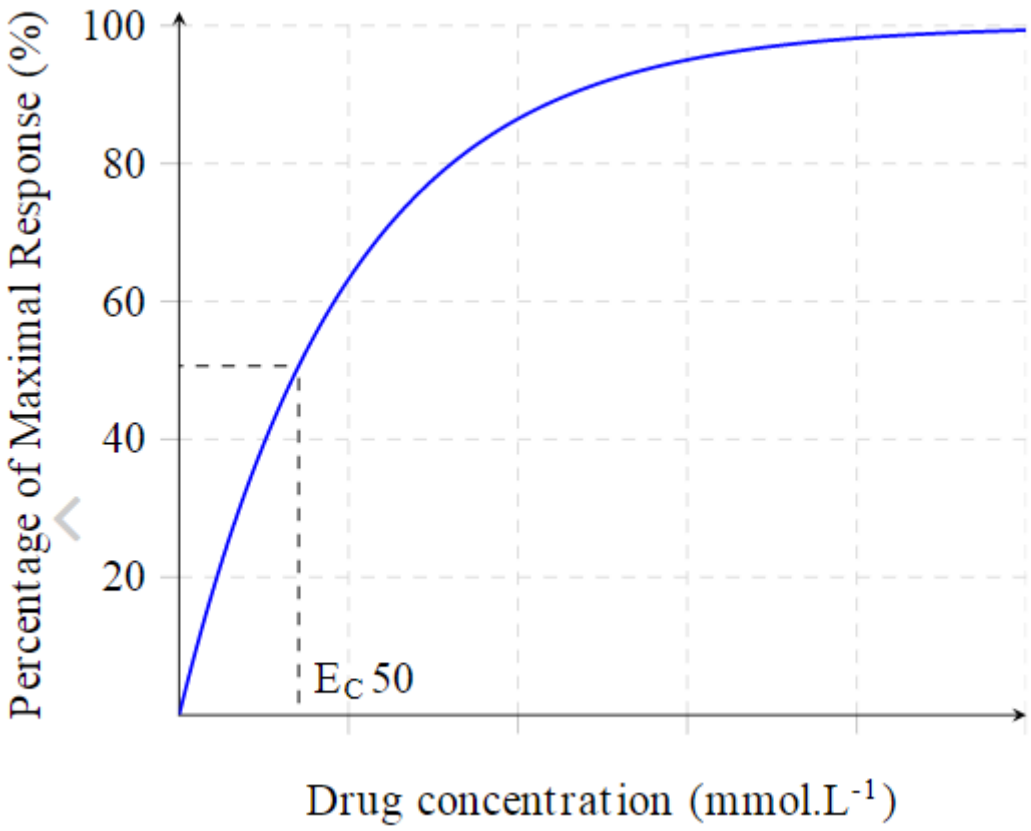
APPENDIX B

Labelled diagram of a Daphnia Magna under a microscope (Sharamok, et al., 2017)



APPENDIX C

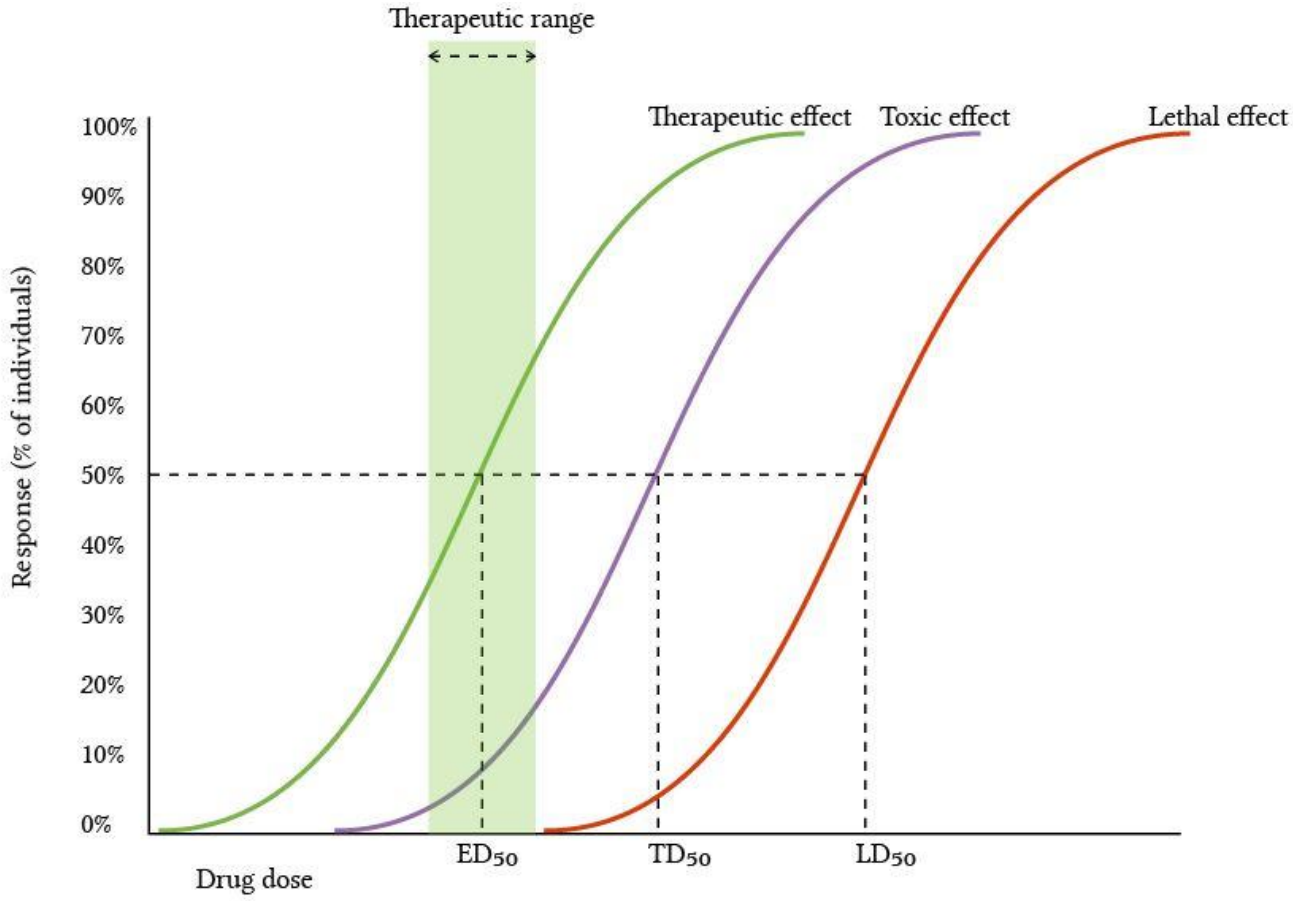
Standard dose response curve showing the effectiveness of a drug (Partone, 2017)



Above is a diagram graph of a standard dose response curve. The curve shows the relation between the drug's effectiveness (response to the drug) against its dose (measured in Millimoles Per Litre).

APPENDIX D

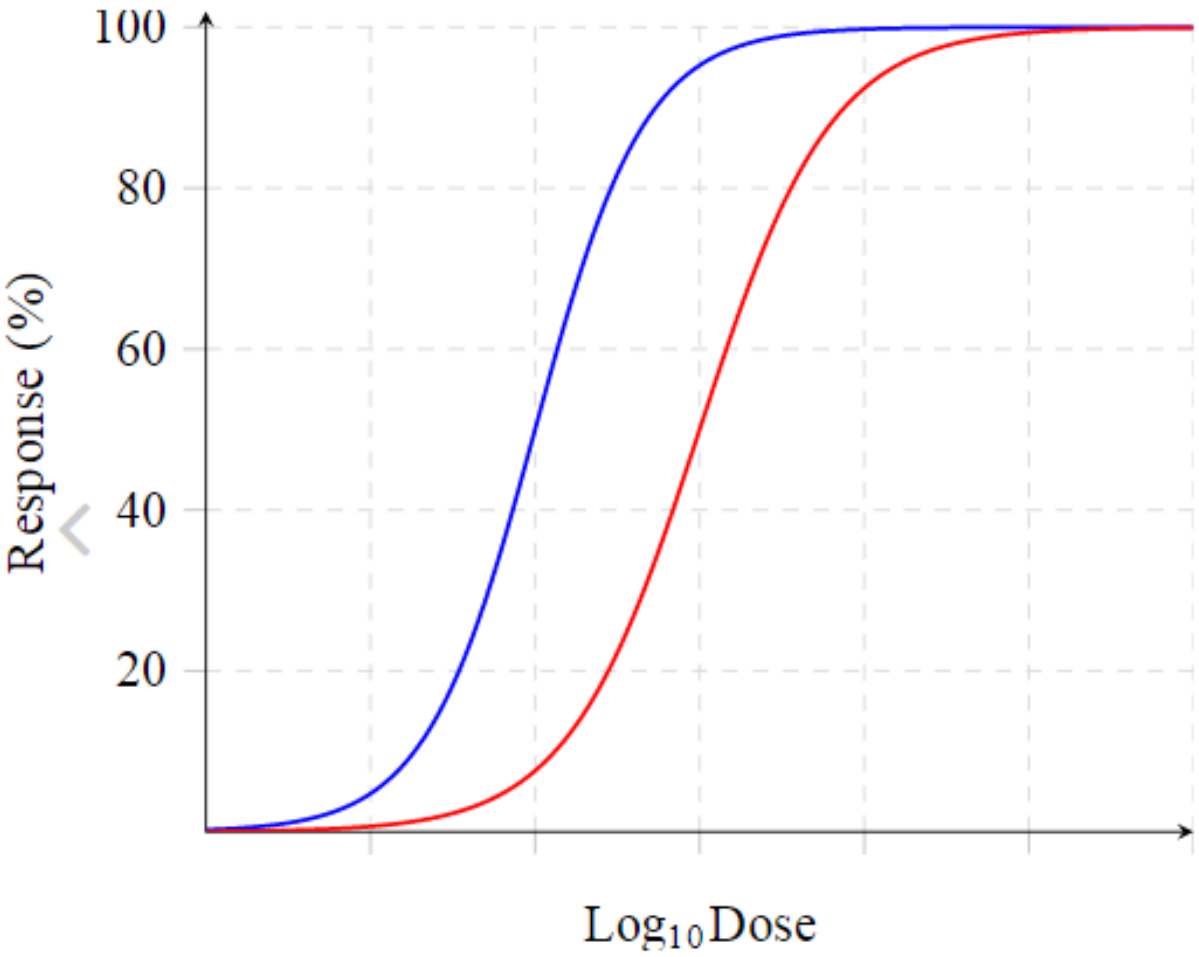
Log-dose response curves (Pharmacodynamics, 2019)



Above is a diagram graph of a quanta dose -response curve (Pharmacodynamics, 2019). The above graph shows the drug dosage on its horizontal axis (X axis) and the response to the drug on its vertical axis (Y axis). The above graph shows the correlation between drug dose and the response to the drug (dose-response relationship) in the form of its median effective dose (ED_{50}), median toxic dose (TC_{50}) and median lethal dose (LD_{50}) (Thomas, et al., 2019) . As seen in the above graph the response to the drug increases at a relatively gradual rate as the dosage of the drug is increased. As seen in log-dose response curves, the curves will plateau at the bottom and top of its vertical axis (0% response and 100% response). This graph shows at what dosage range the drug will generate an effective response without it becoming toxic or lethal, known by and labelled in the above diagram as the therapeutic range.

APPENDIX E

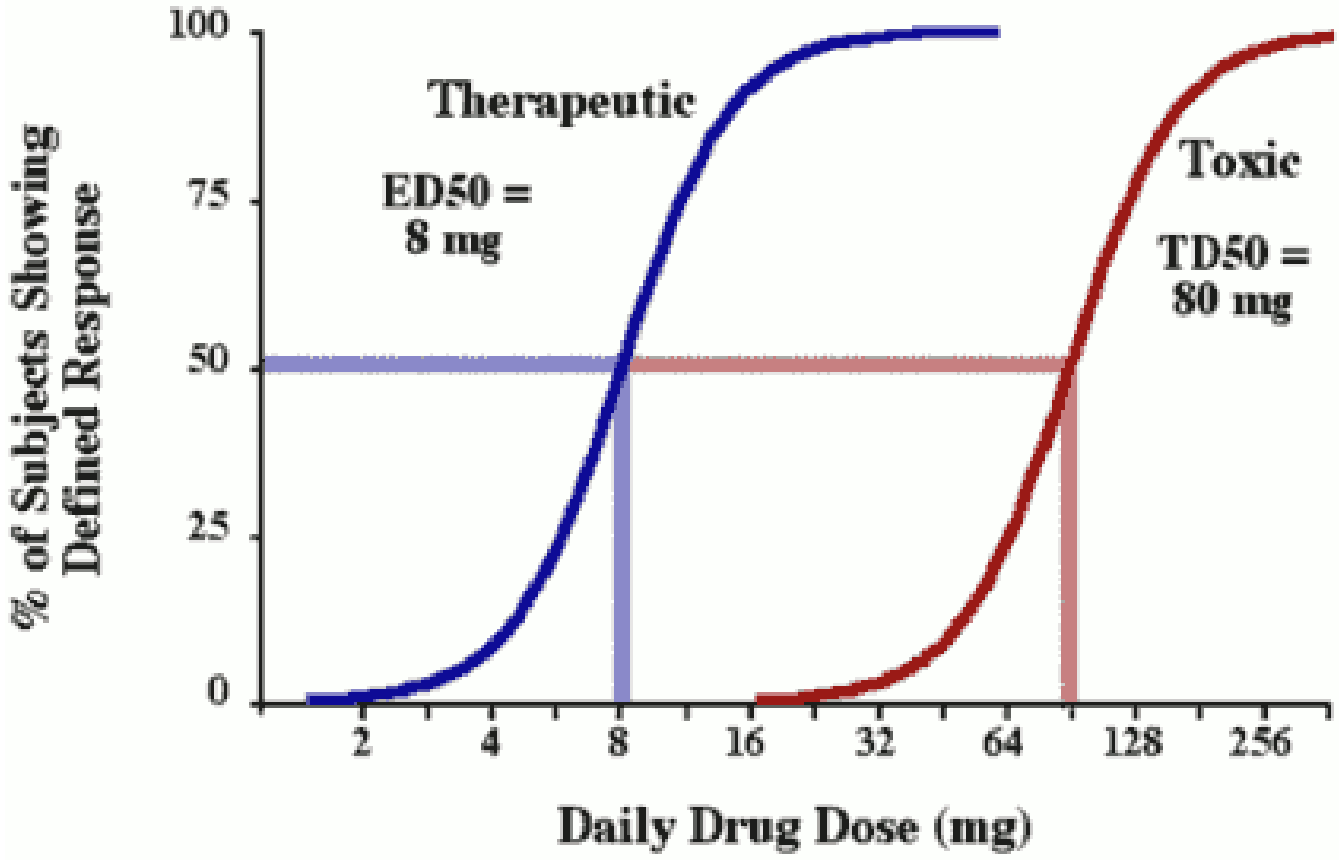
Log-dose response curve showing the effectiveness of two different drugs (Partone, 2017)



The above dose response curve shows the effectiveness of two different drugs against one another. As shown, the drug represented by the blue curve is more potent then the drug represented by the red curve. The drug represented by the blue drug is more potent as it takes a smaller dose to achieve its median effective dose, whereas the drug repreesented by the red curve needs a larger dose to achieve its threshold and maximal effect.

APPENDIX F

Dose response curve (Hecht, n.d.)



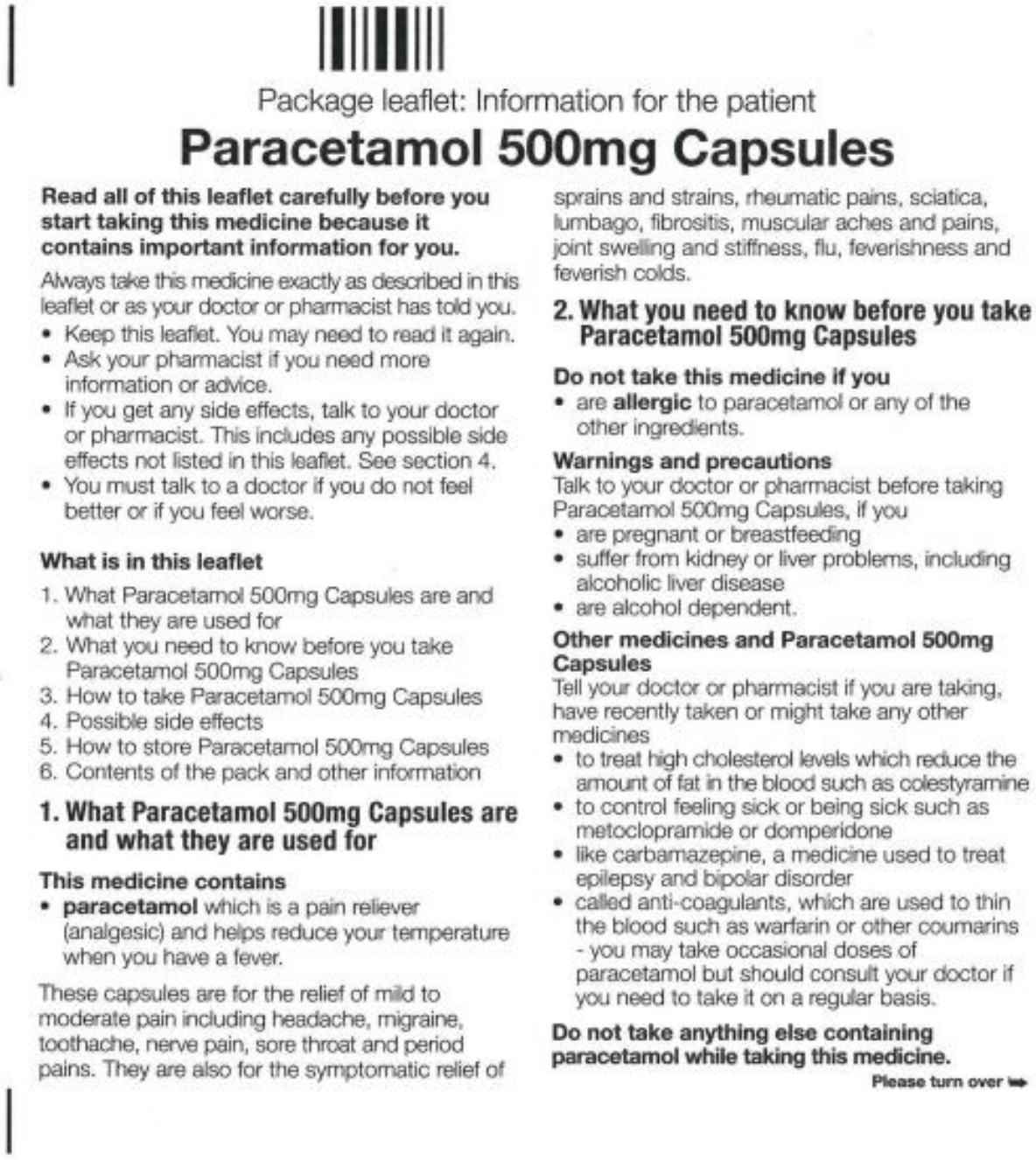
The above dose response curves shows the difference range in which a dose goes from being effective to a dose when it becomes toxic. This margin between the median effective dose (ED_{50}) and the median toxic dose (TD_{50}) is known either as the margin of saftey, or a drugs therapeutic index. The theraputic index is calculated by dividing the median effective dose by its median toxic dose.

$$TI = \frac{Median\ Toxic\ Dose\ (TD_{50})}{Median\ Effective\ Dose\ (ED_{50})}$$

The greater a drugs theraputic index, the safer the drug is as more of the drug is needed for it to reach a toxic level and display adverse side effects. The theraputic index (TI) for the drug shown in the diagram is 10, making it a relatively safe drug.

Dose response curves are often used to calculate a drugs therapeutic index.

APPENDIX G



Package leaflet: Information for the patient

Paracetamol 500mg Capsules

Read all of this leaflet carefully before you start taking this medicine because it contains important information for you.

Always take this medicine exactly as described in this leaflet or as your doctor or pharmacist has told you.

- Keep this leaflet. You may need to read it again.
- Ask your pharmacist if you need more information or advice.
- If you get any side effects, talk to your doctor or pharmacist. This includes any possible side effects not listed in this leaflet. See section 4.
- You must talk to a doctor if you do not feel better or if you feel worse.

What is in this leaflet

1. What Paracetamol 500mg Capsules are and what they are used for

This medicine contains

- **paracetamol** which is a pain reliever (analgesic) and helps reduce your temperature when you have a fever.

These capsules are for the relief of mild to moderate pain including headache, migraine, toothache, nerve pain, sore throat and period pains. They are also for the symptomatic relief of sprains and strains, rheumatic pains, sciatica, lumbago, fibrositis, muscular aches and pains, joint swelling and stiffness, flu, feverishness and feverish colds.

2. What you need to know before you take Paracetamol 500mg Capsules

Do not take this medicine if you

- are **allergic** to paracetamol or any of the other ingredients.

Warnings and precautions

Talk to your doctor or pharmacist before taking Paracetamol 500mg Capsules, if you

- are pregnant or breastfeeding
- suffer from kidney or liver problems, including alcoholic liver disease
- are alcohol dependent.

Other medicines and Paracetamol 500mg Capsules

Tell your doctor or pharmacist if you are taking, have recently taken or might take any other medicines

- to treat high cholesterol levels which reduce the amount of fat in the blood such as colestyramine
- to control feeling sick or being sick such as metoclopramide or domperidone
- like carbamazepine, a medicine used to treat epilepsy and bipolar disorder
- called anti-coagulants, which are used to thin the blood such as warfarin or other coumarins - you may take occasional doses of paracetamol but should consult your doctor if you need to take it on a regular basis.

Do not take anything else containing paracetamol while taking this medicine.

Please turn over ➥

Above is the front scanned page of the information leaflet from the paracetamol capsules used in the experiment. The front of this information leaflet shows side effects of the drug, uses for the drug and warning and precautions. The back of the information leaflet is shown in Appendix H.

APPENDIX H

3. How to take Paracetamol 500mg Capsules

Swallow the capsules whole with water.
Do not chew.

Adults, the elderly and children over 16 years
2 capsules to be taken every 4 to 6 hours, as required. Do not take more than 4 doses (8 capsules) in any 24 hour period.

Children aged 12 to 15 years
1 capsule to be taken every 4 to 6 hours, as required. Do not take more than 4 doses (4 capsules) in any 24 hour period.

Do not give to children under 12 years.

Do not take more often than every 4 hours.
Do not take for more than 3 days unless instructed by your doctor.

Do not take more medicine than the label tells you to. If you do not get better, talk to your doctor.

Talk to a doctor at once if you take too much of this medicine even if you feel well. This is because too much paracetamol can cause delayed, serious liver damage. Go to your nearest hospital casualty department. Take your medicine and this leaflet with you.

4. Possible side effects

Most people do not have any side effects while taking this medicine. However, if you experience any of the following side effects, or anything else unusual happens, stop taking the medicine immediately, and see your doctor or pharmacist.

Rare side effects are
- allergic reactions such as skin rash
- mouth sores
- fever
- difficulty breathing
- you may become more prone to bleeding, bruising, fever and infections, such as sore throat and ulcers, due to changes in your blood
- nausea, sudden weight loss, loss of appetite and yellowing of the eyes and skin.

Very rare cases of serious skin reactions have been reported.

Reporting of side effects
If you get any side effects, talk to your doctor, pharmacist or nurse. This includes any possible side effects not listed in this leaflet. You can also report side effects directly via the Yellow Card Scheme at: www.mhra.gov.uk/yellowcard.
By reporting side effects you can help provide more information on the safety of this medicine.

5. How to store Paracetamol 500mg Capsules

Keep this medicine out of the sight and reach of children. Do not use this medicine after the expiry date printed on the pack.
Do not store above 25°C.

6. Contents of the pack and other information

What Paracetamol 500mg Capsules contain
Each blue / white capsule contains the active ingredient: paracetamol 500mg.

The other ingredients are: maize starch, croscarmellose sodium, sodium laurilsulfate and magnesium stearate. The capsule shell is made of gelatin and contains the colours titanium dioxide (E171), indigo carmine (E132) and erythrosine (E127).

This product is available in pack sizes of 16 and 32 capsules.

The Marketing Authorisation holder and manufacturer is Wrafton Laboratories Limited, Braunton, Devon, EX33 2DL, UK.

Text revised: December 2016.

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Above is the scanned back page of the information leaflet from the paracetamol capsules used in the experiment. This page of the information leaflet shows side effects of the drug, recommended dosages, storage information and contents including additives which were filtered out of the dissolved solution to get a pure paracetamol solution. Each paracetamol capsule contained an average of 0.015g of additives (average obtained from 94 capsules). The batch number of the paracetamol is shown to the bottom right. The front page of the information leaflet is shown in Appendix G.

APPENDIX I

Raw data, observations of Daphnia immediately after exposure to the paracetamol solutions

Concentration (Moles per dm^3)	Number of Daphnia	Observations of Daphnia Magna
Control	21	No change
0.1	23	No change
0.05	27	No change
0.025	25	No change
0.0125	31	No change
0.01	18	No change
0.005	28	No change
0.0025	32	No change
0.001	25	No change
0.0005	35	No change
0.00025	23	No change
0.000125	30	No change
0.0001	28	No change

APPENDIX J

Raw Data 2

Raw data, observations of Daphnia 1 hour after exposure to the paracetamol solutions

Concentration (Moles Per dm^3)	Number of Daphnia	Observations of Daphnia Magna
Control	21	0 dead - No changes in movement
0.1	23	19 dead - Remaining are slow with long breaks between movements
0.05	27	15 dead - Remaining are slow with long breaks between movements
0.025	25	8 dead - Some are slightly slow with others showing no change
0.0125	31	0 dead - Some slow, others show no change
0.01	18	0 dead - Few are slow, others show no change
0.005	28	0 dead - No changes in movement
0.0025	32	0 dead - No changes in movement
0.001	25	0 dead - No changes in movement
0.0005	35	0 dead - No changes in movement
0.00025	23	0 dead - No changes in movement
0.000125	30	0 dead - No changes in movement
0.0001	28	0 dead - No changes in movement

APPENDIX K

Raw Data 3

Raw data, observations of Daphnia 3 hours after exposure to the paracetamol solutions

Concentration (Moles per dm^3)	Number of Daphnia	Observations of Daphnia Magna
Control	21	1 dead - Remaining show no changes in movement
0.1	23	23 dead - All Daphnia deceased
0.05	27	27 dead - All Daphnia deceased
0.025	25	21 dead - Remaining are slow, with long breaks in movemenes
0.0125	31	11 dead - Most remaing are slow with long breaks between movements
0.01	18	3 dead - Some remaining are slow with moderate Periods of no movement
0.005	28	2 dead - Some remaining are slow with short periods of no movement
0.0025	32	0 dead - Some Daphnia are slow with short periods of no movement
0.001	25	0 dead - Some are slow with short periods of no movement
0.0005	35	0 dead - No change in movement
0.00025	23	0 dead - No change in movement
0.000125	30	0 dead - No change in movement
0.0001	28	1 dead - Remaining Daphnia show no change in movement

APPENDIX L

Raw Data 4

Raw data, observations of Daphnia 48 hours after exposure to the paracetamol solutions

Concentration (Moles per dm^3)	Number of Daphnia	Observations of Daphnia Magna
Control	21	2 dead - 3 of the remaining Daphnia are slow. Others show no change
0.1	23	23 dead - All Daphnia deceased
0.05	27	27 dead - All Daphnia deceased
0.025	25	25 dead - All Daphnia deceased
0.0125	31	31 dead - All Daphnia deceased
0.01	18	18 dead - All Daphnia deceased
0.005	28	20 dead - Remaining Daphnia are very slow with long periods of no movement
0.0025	32	21 dead - Remaining Daphnia are slow with moderate periods of no movement
0.001	25	17 dead - Remaining Daphnia are slow with moderate periods of no movement
0.0005	35	17 dead - Most remaining Daphnia are slow, with some periods of no movement
0.00025	23	8 dead - Some remaining Daphnia are slow with some periods of no movement
0.000125	30	16 dead - A few Daphnia are slow, others show no changes
0.0001	28	3 dead - Almost all remaining Daphnia show no changes

Concentration (Moles per dm3)	Dosage (Mg per 50ml)	Dosage (Mcg per 50ml)	*Obsservations of Daphnia Magna*			
			Immediately After Exposure	*1 Hour After Exposure*	*3 Hours After Exposure*	*48 Hours After Exposure*
Control	0.0	0	No change in behaviour or movement	No change in behaviour or movement 0% of Daphnia deceased	5% of Daphnia deceased. Remaining Daphnia show no change in their behaviour or movement	10% of Daphnia deceased. 14% appear slightly slower in movement, with the others showing no change in movement
0.1	755.8	755,825	No change in behaviour or movement	83% of Daphnia deceased. Remaining Daphnia appear slow with little movement and long intervals between movement	100% of Daphnia deceased	100% of Daphnia deceased
0.05	377.9	377,913	No change in behaviour or movement	56% of Daphnia deceased. Remaining Daphnia show little movement with long intervals between movement.	100% of Daphnia deceased	100% of Daphnia deceased
0.025	189.0	188,956	No change in behaviour or movement	32% of Daphnia deceased. Some remaining have very slightly delayed movement whilst others show no change in movement	84% of Daphnia deceased. Remaining Daphnia are very slow with long breaks between movement	100% of Daphnia deceased
0.0125	94.5	94,478	No change in behaviour or movement	0% of Daphnia deceased, some Daphnia appear slow with little movement	35% of Daphnia deceased. Most remaining Daphnia appear slow with moderate periods of no movement	100% of Daphnia deceased
0.01	75.6	75,583	No change in behaviour or movement	0% of Daphnia deceased, few Daphnia appear slow with the majority showing no change in their behaviour or movement	17% of Daphnia deceased. Some remaining Daphnia appear slow with moderate peroids of no movements	100% of Daphnia deceased
0.005	37.8	37,791	No change in behaviour or movement	No change in behaviour or movement 0% of Daphnia deceased	7% of Daphnia deceased. Some of the remaining Daphnia appear slow with some short periods of no movement	71% of Daphnia deceased. Remaining Daphnia are very slow with long periods of no movement
0.0025	18.9	18,896	No change in behaviour or movement	No change in behaviour or movement 0% of Daphnia deceased	0% of Daphnia deceased. Some of the Daphnia appear slow with short periods of no movement	66% of Daphnia deceased. Remaining Daphnia are slow with moderate periods of no movement
0.001	7.6	7,558	No change in behaviour or movement	No change in behaviour or movement 0% of Daphnia deceased	0% of Daphnia deceased. Some of the Daphnia appear slow with occasional short periods of no movement	68% of Daphnia deceased. Remaining Daphnia are slow with moderate periods of no movement
0.0005	3.8	3,779	No change in behaviour or movement	No change in behaviour or movement 0% of Daphnia deceased	0% of Daphnia deceased No change in behaviour or movement	49% of Daphnia deceased. Most remaining Daphnia appear slow with some periods of no movement
0.00025	1.9	1,890	No change in behaviour or movement	No change in behaviour or movement 0% of Daphnia deceased	0% of Daphnia deceased No change in behaviour or movement	35% of Daphnia deceased. Some remaining Daphnia appear slow with some periods of no movement
0.000125	0.9	945	No change in behaviour or movement	No change in behaviour or movement 0% of Daphnia deceased	0% of Daphnia deceased No change in behaviour or movement	53% of Daphnia deceased. Few of the Daphnia appear slower in movement with others showing no changes
0.0001	0.8	756	No change in behaviour or movement	No change in behaviour or movement 0% of Daphnia deceased	4% of Daphnia deceased. Remaining Daphnia show no change in their behaviour or movement	11% of Daphnia deceased. Almost all remaining Daphnia show no changes in their movements or behaviour

APPENDIX N

Immediately After Exposure	
Dosage (Mg per 50ml)	*Population Deceased (%)*
0.0	0
755.8	0
377.9	0
189.0	0
94.5	0
75.6	0
37.8	0
18.9	0
7.6	0
3.8	0
1.9	0
0.9	0
0.8	0

1 Hour After Exposure	
Dosage (Mg per 50ml)	*Population Deceased (%)*
0.0	0
755.8	83
377.9	56
189.0	32
94.5	0
75.6	0
37.8	0
18.9	0
7.6	0
3.8	0
1.9	0
0.9	0
0.8	0

3 Hours After Exposure	
Dosage (Mg per 50ml)	*Population Deceased (%)*
0.0	5
755.8	100
377.9	100
189.0	84
94.5	35
75.6	17
37.8	7
18.9	0
7.6	0
3.8	0
1.9	0
0.9	0
0.8	4

48 Hours After Exposure	
Dosage (Mg per 50ml)	*Population Deceased (%)*
0.0	10
755.8	100
377.9	100
189.0	100
94.5	100
75.6	100
37.8	71
18.9	66
7.6	68
3.8	49
1.9	35
0.9	53
0.8	11

The above tables show the percentage of Daphnia fatalities at each time frame for each solution. The solution concentrations were converted from Moles per cubic decimetre (Moles per dm^3) to milligrams per 50ml of solution (Mg per 50ml).

Concentration (Moles per dm3)	Dosage (Mg per 50ml)	Dosage (Mcg per 50ml)	*Obsservations of Daphnia Magna*			
			Immediately After Exposure	*1 Hour After Exposure*	*3 Hours After Exposure*	*48 Hours After Exposure*
Control	0.0	0	No change in behaviour or movement	No change in behaviour or movement 0% of Daphnia deceased	5% of Daphnia deceased. Remaining Daphnia show no change in their behaviour or movement	10% of Daphnia deceased. 14% appear slightly slower in movement, with the others showing no change in movement
0.1	755.8	755,825	No change in behaviour or movement	83% of Daphnia deceased. Remaining Daphnia appear slow with little movement and long intervals between movement	100% of Daphnia deceased	100% of Daphnia deceased
0.05	377.9	377,913	No change in behaviour or movement	56% of Daphnia deceased. Remaining Daphnia show little movement with long intervals between movement.	100% of Daphnia deceased	100% of Daphnia deceased
0.025	189.0	188,956	No change in behaviour or movement	32% of Daphnia deceased. Some remaining have very slightly delayed movement whilst others show no change in movement	84% of Daphnia deceased. Remaining Daphnia are very slow with long breaks between movement	100% of Daphnia deceased
0.0125	94.5	94,478	No change in behaviour or movement	0% of Daphnia deceased, some Daphnia appear slow with little movement	35% of Daphnia deceased. Most remaining Daphnia appear slow with moderate periods of no movement	100% of Daphnia deceased
0.01	75.6	75,583	No change in behaviour or movement	0% of Daphnia deceased, few Daphnia appear slow with the majority showing no change in their behaviour or movement	17% of Daphnia deceased. Some remaining Daphnia appear slow with moderate peroids of no movements	100% of Daphnia deceased
0.005	37.8	37,791	No change in behaviour or movement	No change in behaviour or movement 0% of Daphnia deceased	7% of Daphnia deceased. Some of the remaining Daphnia appear slow with some short periods of no movement	71% of Daphnia deceased. Remaining Daphnia are very slow with long periods of no movement
0.0025	18.9	18,896	No change in behaviour or movement	No change in behaviour or movement 0% of Daphnia deceased	0% of Daphnia deceased. Some of the Daphnia appear slow with short periods of no movement	66% of Daphnia deceased. Remaining Daphnia are slow with moderate periods of no movement
0.001	7.6	7,558	No change in behaviour or movement	No change in behaviour or movement 0% of Daphnia deceased	0% of Daphnia deceased. Some of the Daphnia appear slow with occasional short periods of no movement	68% of Daphnia deceased. Remaining Daphnia are slow with moderate periods of no movement
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0.00025	1.9	1,890	No change in behaviour or movement	No change in behaviour or movement 0% of Daphnia deceased	0% of Daphnia deceased No change in behaviour or movement	35% of Daphnia deceased. Some remaining Daphnia appear slow with some periods of no movement
0.000125	0.9	945	No change in behaviour or movement	No change in behaviour or movement 0% of Daphnia deceased	0% of Daphnia deceased No change in behaviour or movement	53% of Daphnia deceased. Few of the Daphnia appear slower in movement with others showing no changes
0.0001	0.8	756	No change in behaviour or movement	No change in behaviour or movement 0% of Daphnia deceased	4% of Daphnia deceased. Remaining Daphnia show no change in their behaviour or movement	11% of Daphnia deceased. Almost all remaining Daphnia show no changes in their movements or behaviour

APPENDIX O